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(54) Title: EX VIVO GENERATION OF FUNCTIONAL OSTEOCLASTS FROM BONE MARROW IN A THREE-DIMENSIONAL BIOREACTOR

(57) Abstract: The present invention provides cultured osteoclasts which function to resorb bone and methods for generating such osteoclasts. The method comprises isolating hemopoietic cells or accessory cells, culturing the hemopoietic cells or accessory cells in a chamber having a scaffolding covered or surrounded with culture medium, wherein the scaffolding allows for hemopoietic cells or accessory cells to have cell to cell contacts in three dimensions. The subject osteoclasts are useful for screening drugs which inhibit or stimulate osteoclastogenesis and/or osteoclast function. The subject osteoclasts are also useful in treating certain bone diseases and for use in bone remodeling and regeneration.

EX VIVO GENERATION OF FUNCTIONAL OSTEOCLASTS FROM BONE MARROW IN A THREE-DIMENSIONAL BIOREACTOR

This application was originally filed as U.S. Provisional Application Serial No. 60/148,814 on August 13, 1999.

The invention described herein was made with United States government support under National Science Foundation contract number BES-963160 and may therefore be subject to certain rights of the U.S. government.

FIELD OF THE INVENTION

The present invention relates to the field of cell culture and in particular, to methodologies and compositions related to cultured osteoclasts.

BACKGROUND OF THE INVENTION

Bone is composed of an organic matrix that is strengthened by deposits of calcium salts. Type I collagen constitutes approximately 95% of the organic matrix; the remaining 5% is composed of proteoglycans and numerous noncollagenous proteins. The crystalline salts deposited in the organic matrix of bone under cellular control are primarily calcium and phosphate in the form of hydroxyapatite [2].

Bone also contains at least four different cell types. Osteoblasts, osteoclasts, and bone lining cells are present on the bone surfaces, whereas, osteocytes permeate the mineralized interior. Osteoblasts are the fully differentiated cells responsible for the production of the bone matrix. Osteoblasts are typical protein-producing cells. They secrete type I collagen and the noncollagenous proteins of the bone matrix. Osteoblasts also regulate the mineralization of bone matrix, although the mechanism(s) is not completely understood [2]. Osteocytes are mature osteoblasts within the bone matrix and are responsible for its maintenance. These cells have the capacity not only to synthesize, but also to resorb matrix to a limited extent [3]. Bone lining cells are flat, elongated, inactive cells that cover bone surfaces that are undergoing neither bone formation nor resorption. Little is known regarding the function of these

cells; however, it has been speculated that bone-lining cells can be precursors for osteoblasts [2].

Osteoclasts are giant multinucleated cells responsible for bone resorption. Bone resorption and bone formation are essential processes for normal bone morphogenesis and calcium homeostasis in the body. Aside from its physiological role, bone resorption plays an important role in pathological disorders such as osteoporosis, metabolic bone diseases, bone fracture, and malignant hypercalcemia.

When resorbing bone, osteoclasts demonstrate a polarity defined by a ruffled border that is composed of extensive interdigitated villi in direct contact with bone. Osteoclasts vary between 40-100 μm in diameter and may contain as many as 10-20 nuclei per cell [5]. Active osteoclasts contain extensive mitochondria, lysosomes, and a well-developed Golgi apparatus. These cells are also mobile cells and frequently migrate across a bony surface during the process of bone resorption [6].

Osteoclasts are derived from the macrophage lineage and, thus, are members of the hemopoietic stem cell family. The regulatory pathway for osteoclastogenesis utilizes many of the early steps found in all macrophagic cell differentiation. Recent reports have shown that the myeloid and B lymphocyte transcription factor, PU.1, plays a critical role in osteoclast formation [7]. Transcription factors c-fos and c-src have also been implicated in osteoclast formation [8]. Other animal and human mutations have further delineated the osteoclast pathway. In particular, impaired production of colony-stimulating factor 1 (CSF-1) by stromal cells in the osteoclast microenvironment results in no osteoclasts and no macrophages [9].

Osteoclastogenesis is but the first step in the process of bone resorption. Cell recruitment to a particular site, attachment of cells to bone, demineralization and matrix degradation, motility and apoptosis are all processes that occur as part of the resorptive process. It is now known that most prototypic stimulators of bone resorption (such as parathyroid hormone, vitamin D_3, many

interleukins, thyroid hormone, tumor necrosis factor alpha, etc.) stimulate bone resorption indirectly. This means there are no receptors on osteoclasts for these molecules. Rather, auxiliary cells, usually osteoblasts or stromal lining cells, receive the resorption signal and then elaborate a soluble factor(s) that stimulates the final stages of differentiation of a pre-osteoclast[10].

The signals that tell an osteoclast where to resorb are ill defined. However, the role of integrin receptors in osteoclasts is well defined. These receptors are composed of two subunits, alpha (α) and beta (β), and have a high specificity for bone matrix molecules such as osteopontin and collagen. One widely expressed pair of subunits in the integrin family, $\alpha_v\beta_3$, is present on osteoclasts. Direct examination has shown that interference with $\alpha_v\beta_3$ arrests osteoclast attachment to bone and blocks bone resorption [6]. Once, the osteoclast has established itself on a denuded bone surface it becomes polarized in its orientation. Special organellar structures such as the ruffled border and clear zones appear. The ruffled border is a highly interdigitated villar structure that provides for a large surface area through which the osteoclast can interact with bone. Dissolution of the mineral matrix of bone uncovers the collagen and non-collagen proteins of the organic matrix, material that must be removed in the course of bone resorption. Tartrate-resistant acid phosphatase (TRAP) is a prominent enzyme of osteoclasts that appears to be involved in bone matrix degradation. TRAP can dephosphorylate the protein osteopontin [11].

Osteoclasts are notoriously difficult to isolate. [15] This difficulty arises due to osteoclast number being lower than other bone cell types, the tendency for osteoclasts to adhere to bone matrix, and osteoclast fragility by comparison to other bone cells. Osteoclasts have successfully been generated in traditional flask cultures of murine hemopoietic cells. The first report that demonstrated multinucleated cells (MNCs) with osteoclastic ultrastructural characteristics, were generated in long-term cultures of feline marrow cells [3]. Thereafter, many investigators extended and applied this bone marrow culture system to other animal species, as well as to humans. The MNCs formed in these cultures had characteristic properties of osteoclasts, such as multinuclearity,

TRAP activity, and responsiveness to hormones. However, in some cases, especially human bone marrow cultures, the MNCs that formed did not have the ability to resorb bone [14].

To overcome these difficulties, researchers have used giant cell tumors of bone, such as osteoclastomas, as a source of mature human osteoclasts that are otherwise impossible to extract from the hard matrix of bone [16]. These cells are not the neoplastic cell type and have a normal phenotype and function. Nonetheless, giant cell tumors of bone such as osteoclastomas are cancerous cell lines with inherent limitations for the study of osteoclastogenesis and the use of osteoclasts in clinical applications.

Recently, mouse soluble osteoclast differentiation factor (sODF) has been used to generate functional osteoclasts from human peripheral blood mononuclear cell (PBMC) cultures [17]. ODF is normally expressed on the plasma membrane of osteoblasts/stromal cells and is a ligand for the osteoclastogenesis inhibitor factor (OCIF). Addition of M-CSF to the PBMC culture was also essential for inducing osteoclastogenesis. In addition, dexamethasone was also required for osteoclast formation in human PBMC cultures but not in cultures of the adherent cell population.

The present invention provides methods and means for generating functional osteoclasts from hemopoietic or accessory cells such as found in bone marrow. Functional osteoclasts are generated in a three-dimensional bioreactor which mimics the bone marrow microenvironment and without the requirement of additives that deviate from conditions *in vivo*.

SUMMARY OF THE INVENTION

The present invention provides methods for generating functional osteoclasts from hemopoietic or accessory cells. Conveniently, the hemopoietic or accessory cells may be isolated or derived from bone marrow cells, stroma cells, peripheral blood cells, stem cells, umbilical cord cells, embryonic stem sells peripheral stem cells or any combination of such cells. The method comprises isolating hemopoietic cells or accessory cells and culturing the hemopoietic cells

or accessory cells in a chamber or container having a scaffolding covered or surrounded with culture medium. The scaffolding allows for hemopoietic cells or accessory cells to have cell to cell contacts in three dimensions.

The hemopoietic cells or accessory cells are preferably mammalian hemopoietic cells or accessory cells. In a more preferred embodiment, the mammalian hemopoietic cells or accessory cells are human hemopoietic cells or accessory cells.

In accordance with the present invention, the hemopoietic cells or accessory cells may be mononuclear cells which are those cells left behind when mature red blood cells are removed from the cell sample. Also in accordance with the present invention, the hemopoietic cells or accessory cells are selected from a the group consisting of bone marrow cells, stroma cells, peripheral blood cells, stem cells, umbilical cord cells, embryonic stem cells, peripheral stem cells and any combination of those cells.

The scaffolding for use in the chamber or container may consist of tangled fibers, porous particles, or a sponge-like material. The scaffolding may be formed from a material selected from the group consisting of a synthetic polymer, a natural substance, and a semisynthetic material and may be degradable or non-degradable.

In accordance with the present invention, osteoclasts are generated without the need for additives not found in the *in vivo* bone marrow environment. If desired however, the culture medium may be supplemented to include e.g., 1,25 dihydroxyvitamin D3, its biologically active derivatives and/or other stimulatory factors such as IL-1, TNF, CSF-1, IL-6, lymphotoxin, osteoclast differentiation factor, parathyroid hormone, retinoids, thyroid hormones, and leukotrienes.

The present invention also provides a method for generating functional osteoclasts from hemopoietic or accessory cells by isolating hemopoietic cells or accessory cells, culturing the hemopoietic cells or accessory

cells in a chamber or container having a scaffolding covered or surrounded with culture medium and further identifying cells which resorb bone.

In addition, there is provided a method for generating functional osteoclasts from hemopoietic or accessory cells by isolating hemopoietic cells or accessory cells, culturing the hemopoietic cells or accessory cells in a chamber or container having a scaffolding covered or surrounded with culture medium and further reseeding the chamber with hemopoietic cells or accessory cells.

Osteoclasts cultured from hemopoietic cells and/or accessory cells made in accordance with the methods of the present invention are also provided. Such osteoclasts may be used in bone engineering including bone regeneration, bone synthesis, bone modification or bone remodeling. Thus, in accordance with the present invention, a method of bone engineering is provided which comprises isolating hemopoietic cells or accessory cells, culturing said cells in a chamber having scaffolding covered or surrounded with culture medium, wherein said scaffolding allows for hemopoietic or accessory cells to have cell to cell contacts in three dimensions, and then transplanting the cells with or without support. Osteoclasts may be transplanted with other bone cells such as osteoblasts, together or in series.

The present invention also provides a method of screening for drugs which affect the ability of osteoclasts to resorb bone. The method comprises isolating hemopoietic cells or accessory cells, culturing the hemopoietic cells or accessory cells in a chamber or container having a scaffolding covered or surrounded with culture medium, wherein said scaffolding allows for hemopoietic cells or accessory cells to have cell to cell contacts in three dimensions, isolating osteoclasts from the container; and determining the ability of the osteoclasts to resorb bone in the presence of a test compound. In accordance with this method, drugs which increase or decrease the ability of human osteoclasts to resorb bone are identified. The hemopoietic cells or accessory cells may be mammalian hemopoietic cells or accessory cells.

In a preferred embodiment, the mammalian hemopoietic cells or accessory cells are human hemopoietic cells or accessory cells.

The culture medium for use in the method for screening for drugs which affect bone resorption by osteoclasts may, if desired, include 1,25 dihydroxyvitamin D3 or its biologically active derivatives. In addition, the chamber or container for use in the method for screening drugs which affect bone resorption by osteoclasts may be reseeded with hemopoietic cells or accessory cells.

Also provided by the present invention are isolated osteoclasts which resorb bone. In a preferred embodiment, the isolated osteoclasts have calcitonin receptors. In another preferred embodiment, the osteoclasts express tartrate resistant acid phosphatase. The cultured osteoclasts are useful for treating different conditions associated with under-reacting osteoclasts. The subject osteoclasts may be transfected with a nucleotide sequence of a gene.

A method of identifying genes which are related to osteoclast formation or function is also provided. The method comprises: isolating hemopoietic cells or accessory cells; culturing the hemopoietic cells or accessory cells in a chamber having a scaffolding covered or surrounded with culture medium, wherein said scaffolding allows for hemopoietic cells or accessory cells to have cell to cell contacts in three dimensions; altering one or more culture conditions in a test culture; determining osteoclast number and function in the test sample; and screening for the gene or genes associated with the change in osteoclast number or function in the test sample. Genes may be screened using many different well known methods such as e.g., differential gene display, RNA arbitrarily primed (RAP)-PCR, or gene microarray analysis.

The present invention also provides a method for screening for drugs which affect osteoclastogenesis. The method comprises: isolating hemopoietic cells or accessory cells; culturing the hemopoietic cells or accessory cells in a container or chamber having a scaffolding covered or surrounded with culture medium, wherein said scaffolding allows for hemopoietic cells or

accessory cells to have cell to cell contacts in three dimensions; adding a test compound to the bioreactor, removing cultured cells; and determining the ability of a test compound of affect osteoclastogenesis.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1a is a schematic drawing of one possible configuration of a bioreactor. In the configuration pictured here, the porous or fibrous scaffolding is located in the culture chamber.

Figure 1b is a scanning electron micrograph of a macroporous cellulose microsphere used as artificial scaffolding in the bioreactor.

Figure 2 is a photomicrograph of an "osteoclast-like" cell in Wright's stained cytospin slides from the vitamin D_3-supplemented three-dimensional cultures. The "osteoclast-like" cell appeared to have the features of a giant multinucleated cell, resembling those observed in bone marrow smears.

Figure 3a is a photomicrograph of "osteoclast-like" cells in paraffin thin-sections after culture in the three-dimensional bioreactor.

Figure 3b is a photomicrograph at the same magnification of osteoclast-like cells displaying a polarity defined by a ruffled border, which appears to remain in direct contact with the surface of the matrix (M). The cultures were supplemented with vitamin D_3.

Figure 4 is a photomicrograph of TRAP-positive multinucleated (3 or more nuclei) "osteoclast-like" cells in the three-dimensional mimicry. The cultures were supplemented with vitamin D_3.

Figure 5a is a photomicrograph of the results of a bone resorption assay for the cells harvested from the 3-D bioreactor. Resorption pits formed by the osteoclasts are clearly visible. The solid lines identify the margin of the resorption lacunae.

Figure 5b is a photomicrograph of a bone wafer from a negative control of the same bone resorption assay pictured in Figure 5a (in the absence of vitamin D_3 and PTH).

Figure 6 graphically depicts the effects of vitamin D_3 concentration and PTH on bone resorption activity. PTH concentration used was 10^{-8} M. The data represent averages from triplicate bone wafers. The error bars represent standard deviations. The asterisks indicate the experimental groups where duplicate, instead of triplicate, bone wafers were scored.

Figures 7a and 7b are scanning electron micrographs of bone wafer surface after resorption by osteoclasts generated in the bioreactor. The arrows indicate the remnants of the osteoclast cell.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of culturing osteoclasts from hemopoietic cells or accessory cells. The method comprises isolating hemopoietic cells and/or accessory cells and culturing such cells in a chamber or container having a scaffolding covered or surrounded in culture medium wherein the scaffolding allows for the hemopoietic and/or accessory cells to have cell to cell contacts in three dimensions. It has been surprisingly found that the osteoclasts generated by the methods of the present invention function to resorb bone.

As used herein, the term "hemopoietic cells" means blood cells and blood forming cells and may include, e.g., stem cells, myeloid cells, lymphoid cells, erythroid cells, progenitor cells, precursor cells and mature blood cells such as erythrocytes, neutrophils, monocytes, macrophages, eosinophils, basophils, megakaryocytes, platelets, natural killer cells, T-cells, B-cells, and plasma cells. As used herein "accessory cell" includes any cell which is non-hemopoietic in nature such as stromal cells. Also as used herein, stromal cells may include such cells as endothelial cells, reticular cells, fat cells and professional antigen presenting cells such as dendritic cells. The hemopoietic or accessory cells may be isolated from many different sources such as e.g., bone marrow cells, stroma

cells , peripheral blood cells, stem cells, umbilical cord cells, embryonic stem cells, peripheral stem cells or any combination of these cells.

In accordance with the present invention, a bioreactor system and method for generating functional osteoclasts is provided. As used herein, "functional osteoclasts" mean osteoclasts which function to resorb bone. The bioreactor of the present invention provides a three-dimensional structure which mimics the natural extracellular matrix and ample surface area of the bone marrow and allows cell to cell interaction at a tissue-like cell density. It is understood that the bioreactor of the present invention may have many different configurations so long as it provides a three-dimensional structure. With respect to the bioreactor, the term "three-dimensional structure" is used interchangeably with the term "scaffolding" .

The bioreactor for use in generating functional osteoclasts comprises a container or vessel having at least one chamber or section with scaffolding located therein. The scaffolding is made of a porous or fibrous substrate. Culture media is placed over or around the porous or fibrous substrate.

Figure 1a illustrates one possible configuration of a bioreactor which may be used to generate functional osteoclasts. In Figure 1, the porous or fibrous scaffolding is located in a lower, culture chamber. It is understood that the bioreactor of the present invention may have any number of configurations so long as it provides a three dimensional structure (scaffolding).

The walls of the container or vessel may comprise any number of materials such as glass, ceramic, plastic, polycarbonate, vinyl, polyvinyl chloride (PVC), metal, etc. Culture medium which will support the growth and differentiation of hemopoietic and/or accessory cells into functional osteoclasts is placed over and/or around the porous or fibrous material.

Many different porous or fibrous materials may be used as scaffolding in the bioreactor such as, e.g., tangled fibers, porous particles, sponge, or sponge-like material. The porous or fibrous scaffolding allows hemopoietic and/or accessory cells to lodge onto, proliferate and differentiate. For purposes of

example only and not limitation, suitable scaffolding substrates may be prepared using a wide variety of materials including natural polymers such as polysaccharides and fibrous proteins, synthetic polymers such as polyamides (nylon), polyesters, polyurethanes and minerals including ceramics and metals, coral, gelatin, polyacrylamide, cotton, glass fiber, corrageenans, and dextrans. Examples of tangled fibers include glass wool, steel wool, and wire or fibrous mesh.

Examples of porous particles include, e.g., beads (glass, plastic, or the like) cellulose, agar, hydroxyapatite, treated or untreated bone, collagen, gels such as Sephacryl, Sephadex, Sepharose, agarose or polyacrylamide. "Treated" bone may be subjected to different chemicals such as e.g., acid or alkali solutions. Such treatment alters the porosity of bone. If desired, the substrate may be coated with an extracellular matrix or matrices, such as, e.g., collagen, matrigel, fibronectin, heparin sulfate, hyalumonic and chondroitin sulfate, laminin, hemonectin, or proteoglycans.

The fibrous or porous material used as scaffolding in the bioreactor forms openings or pores into which hemopoietic and/or accessory cells enter. Once entered, the cells become entrapped or adhered to the fibrous or porous material and colonize and/or aggregate thereon. Cell attachment and colonization can occur merely by inoculating the cells into the culture medium which overlays and/or surrounds the porous or fibrous substrate. Cell attachment and colonization may also occur by inoculating the cells directly onto the porous or fibrous substrates .

In accordance with the present invention, hemopoietic and/or accessory cells must be able to enter the openings (pores) of the fibrous or porous material. The skilled artisan is cognizant of the different sizes of hemopoietic and accessory cells and therefore the pore size needed to accommodate such cells. Generally speaking, a pore size in the range of from about 15 microns to about 1000 microns may be used. Preferably, a pore size in the range of from about 100 microns to about 300 microns is used.

In a preferred embodiment, a membrane is placed in the bioreactor in order to facilitate gas exchange. The membrane is gas permeable and may have a thickness in the range of from about 10 to about 100 μm. In a more preferred embodiment, the membrane has a thickness of about 50 μm. The membrane is placed over an opening in the bottom or side of the chamber or container. In order to prevent excessive leakage of media and cells from the bioreactor, a gasket may be placed around the opening and /or a solid plate placed under or alongside the opening and the assembly fastened.

The cell medium used in the bioreactor may be any of the widely known media used to support growth and differentiation of bone marrow cells, and in particular, growth and differentiation of hemopoietic and/or accessory cells into functional osteoclasts. For example, the following classical media may be used and supplemented, if desired, with vitamin and amino acid solutions, serum, and/or antibiotics: Fisher's medium (Gibco), Basal Media Eagle (BME), Dulbecco's Modified Eagle Media (D-MEM), Iscoves's Modified Dulbecco's Media, Minimum Essential Media (MEM), McCoy's 5A Media, and RPMI Media.

Specialized media may also be used such as e.g., MyeloCult ™ (Stem Cell Technologies), and Opti-Cell ™ (ICN Biomedicals). If desired, serum free media may be used such as , e.g., StemSpan SFEM ™ (StemCell Technologies), StemPro 34 SFM (Life Technologies) and Marrow-Gro (Quality Biological Inc.)

In a preferred embodiment, McCoy's 5A medium (Gibco) is used at about 70% v/v, supplemented with vitamin and amino acid solutions. In an even more preferred embodiment, the culture medium comprises approximately 70% (v/v) McCoy's 5A medium (Gibco), approximately $1x10^{-6}$ M hydrocortisone , approximately 50 ug/ml penicillin, approximately 50 mg/ml streptomycin, approximately 0.2 mM L-glutamine, approximately 0.45% sodium bicarbonate, approximately 1x MEM sodium pyruvate, approximately 1x MEM vitamin solution, approximately 0.4x MEM amino acid solution, approximately

12.5% (v/v) heat inactivated horse serum and approximately 12.5% heat inactivated FBS. The medium chamber may be continuously perfused if desired.

The bioreactor is inoculated with hemopoietic and/or accessory cells by gently adding e.g., pipetting, into the three-dimensional scaffolding portion of the bioreactor. Alternatively, the hemopoietic and/or accessory cells may be added to the culture covering and/or surrounding the three dimensional scaffolding. Cells will settle or migrate into the porous or fibrous material making up the scaffolding. The number of cells added to the bioreactor depends on the total area of the three-dimensional scaffolding and volume of culture media. Preferably, hemopoietic and/or accessory cells isolated from any of the sources discussed extensively herein, are centrifuged through a gradient such as a Ficol/Plaque to remove mature red blood cells, yielding mononuclear cells.

For a bioreactor having a culture chamber of about 3/16" height by about 5/16" width by about 5/16" length and packed with about 0.01 g of a porous or fibrous substrate, the number of mononuclear cells added to the bioreactor may be anywhere in the range of from about 10^4 to 10^9 mononuclear cells. Preferably, 4-6 x 10^6 cells may be used to inoculate the bioreactor. Using these guidelines, one skilled in the art is able to adjust the number of cells used to inoculate the bioreactor depending on the total area of the three-dimensional scaffolding, volume of culture media, type of three-dimensional scaffolding, and source of hemopoietic and/or accessory cells.

Preferably, the culture is fed every second day with the culture medium. Various other ingredients may be added to the culture media in order to further stimulate osteoclast growth and differentiation. Thus for example, vitamin D3 (1, 25 dihydroxyvitamin D3), biologically active derivatives of 1, 25 dihydroxyvitamin D3, osteoclast differentiation factor, parathyroid hormone (PTH), retinoids, thyroid hormones, leukotrienes, interleukin-1, interleukin-6, lymphotoxin, tumor necrosis factor, and colony stimulating factor-1 may be added to the culture media.

In a preferred embodiment, vitamin D3 is used to stimulate osteoclast proliferation, differentiation and function. The cell culture is allowed to grow anywhere from about a few days to a few weeks.. Preferably, the cultures are harvested after about three weeks. Hydrocortisone is also preferably removed from the culture medium anywhere from about one to three weeks to avoid any potential inhibition of osteoclastogenesis.

Cells may be harvested in any number of well known methods. The chamber may be treated with any suitable agent, such as collagenase, to release the adhering cells. Non-adhering cells may be collected as they release into the medium. Cells may also be removed from the substrate by physical means such as shaking, agitation, etc. Thereafter, the cells are collected using any known procedure in the art such as e.g., pipetting or centrifugation. Preferably, non-adherent cells are released by gentle stirring and mixing the bed of porous or fibrous material and then collected by centrifugation or sedimentation.

If desired, the cell samples collected from the bioreactor may be further enriched for osteoclasts using well known methods of positive selection. Thus, for example, a solid support (such as beads) having an antibody that binds osteoclasts conjugated thereto, may be mixed with the cell sample. Antibody conjugated beads with osteoclasts bound thereto are then collected by gravity or other means such as a magnet, in the case of magnetic beads.

Negative selection may also be used as a means of enriching the osteoclast population in the cell sample removed from the bioreactor. With a negative selection scheme, a solid support (such as beads) having conjugated thereto one or more antibodies which react with cells other than osteoclasts may be mixed with the cell sample. Antibody conjugated beads with cells other than osteoclasts bound thereto are then collected by gravity or other means such as a magnet, in the case of magnetic beads.

In either positive or negative selection, the osteoclasts may be further isolated by filtration based on size. In accordance with the present invention, however, the cell samples removed from the bioreactor comprise

functional osteoclasts which may be used in many different clinical and drug screening settings without being further enriched.

Osteoclasts may be identified using any of the well known indicia such as, e.g. multinuclearity, tartate-resistant acid phosphatase (TRAP) activity, the presence of calcitonin (CT) receptors and responsiveness to hormones including 1, 25 – dihydroxyvitamin D_3 [I, 25 $(OH)_2D_3$], parathyroid hormone (PTH) and certain cytokines.

The osteoclasts produced by the methods of the present invention function to resorb bone. The ability of osteoclasts produced by the methods of the present invention to resorb bone may be assayed using well known bone resorbing assays such as those described in Jones et al. 1986 *Scanning Electron. Micros. 4*:1571-1580 and Boyde et al. *Scanning Electron. Micros. 3*:1259-1271, using dentine. or the methods described in Chambers et al. 1985 *Endocrinology 116*:234-239 and Chambers et al. 1984 *J. Cell Sci. 66*:383-399, using bone. A bone resorbing assay especially adapted for use with the cultured osteoclasts of the present invention is described herein at Example 7.

The cultured osteoclasts of the present invention have a myriad of uses in the therapeutic and pharmaceutical industries. For example, the subject osteoclasts may be used to screen for drugs which either inhibit or stimulate osteoclastogenesis or osteoclast function, e.g., bone resorbing activity. As used herein, "osteoclastogenesis" is meant to mean the derivation of osteoclasts from the hemopoietic stem cell family. By "bone resorption" or "bone resorbing activity" is meant any of the steps in bone resorption such as cell recruitment, attachment of cells to bone, demineralization and matrix degradation, motility, and apoptosis.

It is now known that various diseases are associated with either under-reacting or over- reacting osteoclasts. For example, osteoporosis is a condition where bone is resorbed in an excess amount, and osteoclasts are considered over-reactive. Thus, inhibitors of osteoclastogenesis or bone resorption identified by the assays of the present invention are useful for the

treatment of diseases associated with over-reactive osteoclasts such as osteoporosis.

In certain types of metastatic cancers, e.g., breast and prostate cancers, cancer cells may migrate from the primary tumor and lodge in bone. By inhibiting bone resorption by osteoclasts, bone lesions are reduced and the incidence of cancer cells invading bone through such lesions may be reduced.

Various diseases conditions such as osteopetrosis are thought to be caused by under-reactive osteoclasts. Such diseases may be treated with those drugs identified by the subject assays as stimulators of osteoclastogenesis or bone resorption.

Thus in accordance with the present invention, there are provided methods of screening for drugs which affect osteoclastogenesis or the ability of osteoclasts to resorb bone. As used herein, "drug" or "test compound" encompasses any element, molecule, chemical compound, hormone, growth factor, nucleotide sequence (including oligonucleotides), protein (including peptides), and reagents which have the ability to inhibit or stimulate osteoclastogenesis, or osteoclast function.

In a typical screening assay for a drug which affects osteoclast function, e.g, bone resorption, cultured osteoclasts are removed from the bioreactor and placed in a petri dish, flask, microscope slide, microtiter dish or the like with enough culture medium or buffered solution to keep the osteoclasts alive. The liquid medium should preferably mimic the bone environment of the body since this is ultimately where the drug which inhibits or stimulates osteoclast function will be acting. Preferably, a pH of approximately 7.2, and a temperature of about 37 ° C is maintained. If desired, during the screening assay, the cultured osteoclasts of the present invention may be placed on bone or dentine samples in an environment which mimics the human body . The number of osteoclasts which may be used in a screening assay is empirical. Typically, a sample containing $1 X10^{6}$ total cells may be used, depending upon the number of osteoclasts in the cell sample.

The number of osteoclasts in a cell sample relative to other cells may be determined microscopically by counting giant multi-nucleated cells. TRAP staining, bone resorption assays or a combination thereof may also be performed. Methods of cell counting are well known in the art and are also described in Example 4. The concentration of the test compound i.e., drug to be screened as a potential inhibitor or stimulator of osteoclast activity is empirical. One skilled in the art is familiar with methods of adjusting concentrations of different compositions in order to best identify the effects of a test compound in the screening assay. Typically, a range of concentrations is used and those portions of the range which exhibit serious deleterious effects on osteoclast viability eliminated for further study. Those portions of the range having less deleterious effects on osteoclast viability are identified and used for further study of inhibitory or stimulatory effects on osteoclast formation, osteoclast activity or osteoclast functionality.

The mixture of osteoclasts and test compound is incubated for a time and under conditions sufficient for the inhibition or stimulation of bone resorbing activity to be carried out. As defined herein, a sufficient time can be anywhere from about five minutes to several hours or more. When osteoclasts are tested in a petri dish, flask, microscope slide, microtiter dish or the like, a sufficient time may be several minutes to several hours . Of course, the test time may be extended if needed in order to see effects on the cells. The skilled artisan is able to determine the optimal time for running the screening assay by removing samples and examining cells microscopically for viability. Bone resorption assays may then be carried out to determine the effect of the drug on bone resorption. When osteoclasts are tested directly on bone or dentin, the time sufficient for inhibition or stimulation of bone resorbing activity may be extended . For example, the test time may be several days to about ten days or more.

A preferred buffer for use in the reactions is Phenol red-free MEM supplemented with 1 X nonessential amino acids, 1X L-glutamine, 10% FBS, 50 U/ml penicillin and 50 μg/ml streptomycin. In a preferred embodiment, the test reaction volume is between about 0.5 and about 2 ml. In a more preferred

embodiment, the reaction volume is about 1 ml.. In a preferred embodiment, the incubation temperature is approximately 37°C.

In accordance with the present invention, a control assay is preferably performed where the cultured osteoclasts are treated the same as in the test assay with the exception that a test compound is not added to the osteoclasts . After a time sufficient for the test compound to either inhibit or stimulate bone resorption, bone resorption assays are performed. A bone resorption assay may also be performed in the presence of the test compound. Methods for assaying bone resorption by osteoclasts are well known in the art and described herein. By comparing bone resorption between osteoclasts in a control sample and osteoclasts in a test sample, a test compound which inhibits or stimulates bone resorption is identified.

In an alternative embodiment, there is provided a method for screening for drugs which either inhibit or stimulate osteoclastogenesis. In this embodiment, a test compound is added directly to the bioreactor. The test compound may be added to the culture medium or into the three dimensional scaffolding. The time at which the test compound is added is empirical but is relatively early. Typically, control runs are performed in which no test compounds are added to the bioreactor.

The ability of a test compound to inhibit or stimulate osteoclastogenesis may be determined by osteoclast cell count, TRAP staining, bone resorption assays or a combination thereof. Methods of cell counting are well known in the art and are also described in Example 4. Cell counts are compared between experimental and control assays. Increased numbers of osteoclasts compared to control runs correlate with the identification of a stimulator of osteoclastogenesis. Decreased numbers of osteoclasts compared to control runs correlate with the identification of an inhibitor of osteoclastogenesis.

Examples of inhibitory test compounds which may be screened in the assays of the present invention include, e.g., functional analogs of calcitonin, INF-γ, and TGF-β, osteoclastogenesis inhibitor factor (OCIF), as well as various

glucocorticoids, and analogs of estrogen and androgen. Example of stimulatory test compounds which may be screened in the assays of the present invention include, e.g., functional analogs of IL-1, TNF, CSF-1, and IL-6, and osteoclast differentiation factor (ODF). As described above, however, any available test compound may be used to screen for effective inhibitors of osteoclastogenesis and /or osteoclast activity or functionality.

In some cases, the classification of a test compound as potential inhibitor or potential stimulator of osteoclastogenesis or bone resorption is unknown and is initially determined by the assay.

Also in accordance with the present invention, there are provided osteoclasts which have the ability to resorb bone. Thus the osteoclasts of the present invention may be used in treating bone conditions which are associated with under-reacting osteoclasts. Examples of such conditions includes osteopetrosis. On the other hand, Paget's disease is initiated by increased osteoclast-mediated bone resorption followed by a compensatory increase in new bone formation.

Since it is known that bone resorption carried out by osteoclasts is part of both the bone formation and bone remodeling process, the osteoclasts of the present invention may also be used in bone synthesis, bone modification and bone engineering, including preparations of bone and dental prostheses.

In either aspect of the invention, the functional osteoclasts generated in the bioreactor are removed from the bioreactor and transplanted. As used herein, "transplanting" or "transplantation" means removing osteoclasts from the bioreactor for further processing including other culturing for the purposes of *ex vivo* bone regeneration, synthesis, modification or remodeling. As used herein, "transplantation" also encompasses implanting osteoclasts into the human body such as direct transplanting onto bone *in vivo*.

The osteoclasts of the present invention may be transplanted with or without other bone cells such as e.g., osteoblasts. Bone engineering (including regeneration, synthesis, modification or remodeling) using osteoblasts alone

results in the formation of woven bone instead of lamellar bone which is normally found in bone. Thus, current bone engineering capabilities using osteoblasts are improved using the cultured osteoclasts of the present invention in conjunction therewith. Osteoblasts and the subject osteoclasts may be transplanted together or in series. The transplantation may be *in vivo* or *ex vivo*. Bone cells other than the subject osteoclasts may be cultured or isolated from the body.

The subject osteoclasts may be transplanted with or without a support (scaffolding). As is known in the art, there are many different types of scaffolding which are used for bone engineering. Examples include any of the porous or fibrous materials used as scaffolding in the bioreactor as discussed extensively herein. Other examples include titanium coated polymers, and CellFoam™ which is produced by high-temperature precipitation of tantalum as a thin layer onto a highly regular reticulated carbon skeleton.

Thus, the osteoclasts of the present invention may also be transplanted in order to help in bone remodeling or bone regeneration. There are various conditions associated with defects in bone remodeling and the subject osteoclasts may be transplanted *in vivo* or *ex vivo* in order to correct such conditions. The subject osteoclasts used for transplantation may be transfected with a nucleotide sequence of a gene. The gene may be heterologous to the species from which the osteoclasts are generated or native to the species from which the osteoclasts are generated. For example, the subject osteoclasts may be transformed with the nucleotide sequence encoding osteoclast differentiation factor (ODF). The nucleotide sequences for osteoclast differentiation factor are known and readily available.

As described above, the subject osteoclasts may be transplanted with or without other types of bone cells, e.g., osteoblasts. Similarly, the osteoclasts of the present invention may be used in bone regeneration. Thus for example, as a substitute for titanium or plastic bone replacement, the subject osteoclasts may be transplanted onto defective bone, e.g., broken or shattered, or other support in a method of regenerating bone. Again, the subject osteoclasts

may be transplanted with or without other types of bone cells, e.g., osteoblasts., together or in series.

The present invention also provides a method for identifying genes which are related to in osteoclast formation or function. In this aspect of the invention, various parameters of the culture conditions may be changed such as e.g, nutrient ingredients including osteoclastic agents such as osteoclast differentiation factor, temperature, oxygen concentration, CO_2 concentration, and nutrient composition. After altering one or more parameters, osteoclast number and function is determined. The osteoclast number may be determined by counting TRAP positive cells. Osteoclast function may be determined by bone resorption assay. If changes in osteoclast number and function occur in a test sample when compared to a control sample, then the system may be used to further screen for the gene or genes accountable for the change. Differential gene display, its modified versions such as RNA arbitrarily primed (RAP)-PCR technique or gene microarray analysis may be used to further identify and characterize the genes involved. These methods are well known in the art. Furthermore, the genes associated with the osteoclasts of the test sample may be identified by cloning the genes expressed by the purified or enriched subject osteoclasts.

The invention is further illustrated by the following specific examples which are not intended in any way to limit the scope of the invention.

EXAMPLE 1

Preparation of the Bioreactor

The bioreactor was fabricated using polycarbonate plates (Figure 1A). The culture chamber (3/16"H x 5/16"W x 5/16"L) was packed with 0.01 g of the highly porous microcuries. The packed-bed of microcarriers was overlayered with culture medium. The medium chamber (1/2"H x 5/16"W x 12/16"L) contained 0.6 ml of medium. A Teflon™ membrane (50 μm thickness) was used to facilitate gas exchange.

Cellsnow™-EX, type L (low ion-charged), macroporous cellulose microcarriers (Kirin, Japan; 1-2 mm diameter; 100-200 μm pore size; 95% porosity) were used throughout these experiments as an artificial scaffolding for the human bone marrow cells (Figure 1B).

EXAMPLE 2

Human Bone Marrow Preparation

Bone marrow, aspirated from the iliac crest of consenting donors according to the instructions from the University of Rochester's Research Subjects Review Board, was diluted 1:1 with McCoy's 5A medium (Gibco, Grand Island, NY), overlayered onto Ficol/Paque (Pharmacia, Piscataway, NJ, density 1.027 g/ml), and centrifuged at 200 *g* for 30 minutes. The mononuclear cell layer was collected, washed 3 times, and used to inoculate the bioreactor. A portion of the cells was set aside to be used in various assays as needed.

EXAMPLE 3

Three-Dimensional Human Long-Term Bone Marrow Culture

The cultures were inoculated with the proper number of mononuclear cells (4-6 x 10^6 cells per culture chamber) by pipetting into the porous microcarrier section of the bioreactor. The cultures were incubated in a humidified CO_2 incubator (containing 5% CO_2) at 37 °C. The LTBMC medium (changed daily), consisted of 70% (v/v) McCoy's 5A medium (Gibco), 1 x 10^{-6} M hydrocortisone (Sigma, St. Louis, MO), 50 u/ml penicillin (Sigma), 50 mg/ml streptomycin (Sigma), 0.2 mM L-glutamine (Gibco), 0.045% sodium bicarbonate (Sigma), 1x MEM sodium pyruvate (Gibco), 1x MEM vitamin solution (Gibco), 0.4x MEM amino acid solution (Gibco), 12.5% (v/v) heat inactivated horse serum (Gibco), and 12.5% heat inactivated FBS (Gibco). For the first 2 weeks, the cultures were fed every second day with the complete culture medium. At week 2, the cultures were depopulated by gently stirring and mixing the bed of porous microspheres to release the non–adherent cells (50 µl/well). Viable cell count for the nonadherent cells was determined by the dye–exclusion method using Trypan blue dye (Sigma) and a hemocytometer. Commencing on week 2, the cultures were fed with hydrocortisone-free medium supplemented with 1,25-Dihydroxyvitamin D_3 (ICN Biomedicals, Aurora, OH) at different concentrations (10^{-7}, 5 x 10^{-8}, 10^{-8}, and 10^{-9} M) every 2 days. The cultures were harvested at week 3, gentle pipetting and sacrificed at week 4 to perform the various assays.

EXAMPLE 4

Differential Cell Analysis

Cytospin slides of the nonadherent cells obtained from the human LTBMC were prepared by centrifugation of 20,000 cells/slide in cytospin funnels at 500 rpm for 5 min using a cytospin centrifuge (Shandon, Sewickly, PA). The cells were air-dried prior to staining with Wright's stain (Geometric Data, Wayne, PA) for 15 min followed by a distilled water wash for 1 min. Differential cell count was performed blindly by counting over 100 cells per sample. For each culture condition, six to nine identical cultures were established [19]. The presence of morphologically-distinguishable "osteoclast-like" cells was observed in the cell output from both the vitamin D_3-supplemented and non-supplemented cultures (Figure 2). These giant, multinucleated cells exhibited the typical cell morphology of osteoclast cells observed in bone marrow smears and were easily distinguishable from macrophages and megakaryocytes.

EXAMPLE 5

Cell Morphology Characterization

The scaffolding and the cells within removed from the bioreactor were embedded in 2% Bacto agar (Gibco) and then fixed in 10% neutral buffered formalin (Fisher, Pittsburgh, PA) for at least one hour. They were then infiltrated with paraffin [20]. Paraffin thin-sections of the three-dimensional LTBMCs were serially cut at 4-5 μm thickness and mounted on chemically coated slides. The paraffin thin-sections were deparaffinized with xylene, 100% alcohol, 95% alcohol, and 70% alcohol followed by a rinse with tap water. The thin-sections were then stained with Mayers hematoxylin-eosin and microscopically examined in order to characterize cell morphology and confirm[20] the presence of "osteoclast-like" cells. As depicted in Figures 3A and 3B, the cells displayed a polarity defined by a ruffled border, which appeared to remain in direct contact with the surface of the matrix. Thus the three-dimensional scaffolding mimics normal marrow spatial organization and allows cellular interactions with the extracellular matrix, resulting in osteoclast-like cells with a morphology similar to osteoclasts found in bone.

EXAMPLE 6

Cytospin Preparations for TRAP

Cytospin slides with cells harvested from the three-dimensional cultures were prepared as discussed in Example 4. The slides were air dried and kept in the refrigerator until TRAP staining. Prior to TRAP staining, the slides were brought to room temperature. Following the manufacturer's instructions for the Acid Phosphatase, Leukocyte Kit (Sigma, St. Louis, MO), the slides were fixed in the fixation solution (containing formalin, acetone and citrate) for thirty seconds, followed by a wash with distilled water. The slides were then stained in the following prewarmed mixture: Diazotized Fast Garnet GBC Solution, Naphthol AS-BI Phosphate Solution, Acetate Solution with Tartrate Solution for one hour at 37° C and protected from light. Slides were rinsed in distilled water, stained in Gill Hematoxylin for two minutes and blued in 0.3% Ammonia Water for 8 dips [20,21]. Smears were air dried and viewed using a Zeiss Axioscope microscope (Zeiss, Thornwood, NY).

The large, multinucleated "osteoclast-like" cells identified in the Wright's stained cytospin slides, revealed a strong positive reaction (Figure 4) for tartarate-resistant acid phosphatase (TRAP). The majority of these multinucleated cells (containing 3 or more nuclei) stained positive for TRAP. Similar results were obtained in two independent experiments.

EXAMPLE 7

Bone Resorbing Assay

Osteoclast functionality was determined by performing a bone-resorbing assay. Briefly, cells harvested from the three-dimensional bioreactor were cultured on bone wafers (4 mm x 4 mm) from bovine femur cortical bone. The cells were allowed to settle for 30 min onto the wafer. The wafers were then transferred into the wells of 96-well plates and covered with medium. The medium consisted of Phenol red-free MEM (Gibco), 1x Nonessential amino acids (Gibco), 1x L-glutamine (Gibco), 10% heat inactivated FBS (Gibco), 50 U/ml Penicillin, and 50 mg/ml Streptomycin. The medium was supplemented with the appropriate concentration of vitamin D_3, and with, or without, parathyroid hormone (PTH, 10^{-8} M; Sigma). The bone wafers were placed in an incubator (containing 5% CO_2) at 37 °C for an additional 10 days. The wafers were stained with toluene blue and scored for resorption pits using a Reflected Light Microscope (Olympus, Melville, NY). The number and surface area of the osteoclast pits were quantified using a digital imaging software and were an indication of the cell's activity [7]. Triplicates were scored.

In a separate experiment, after 4 weeks of culture in the bioreactor, the cultures were harvested and a bone resorption assay was performed on bone wafers from bovine femur cortical bone. Numerous resorption pits were formed (Figure 5). No resorption pits were formed on the negative controls. The bone wafers were scored for resorption pits. The number and surface area of the osteoclast pits were quantified and were an indication of the cell's activity. A dose response for vitamin D_3 was observed (Figure 6). The high concentration of vitamin D_3 (10^{-7} M) resulted in the highest levels of resorption activity. Moreover, the supplementation with PTH further stimulated the resorption activity. This result is in accordance with the stimulatory role of PTH [23] confirms that the "osteoclast-like" cells produced in the three-dimensional mimicry behave like true osteoclasts.

EXAMPLE 8

Scanning Electron Microscopy (SEM)
Studies of Bone Wafer Surface After Resorption

The bone wafers, which were used for the bone resorption assay (Example 7), were placed on sample stages and then coated with gold in a Desk II Vacuum (Rando, Wyckoff, NJ) [22]. A Leo 982 digital SEM (Leo Electron Microscopy, UK) was used. Scanning electron microscopy of the bone wafers revealed the resorption lacunae excavated by the osteoclasts (Figure 7a and 7b). The micrographs demonstrate that dissolution of the mineral occurred before the degradation of the organic matrix, i.e., the collagen fibers.

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WHAT IS CLAIMED:

1. A method of culturing osteoclasts from hemopoietic cells or accessory cells, comprising:

isolating hemopoietic cells or accessory cells; and

culturing the hemopoietic cells or accessory cells in a chamber having a scaffolding covered or surrounded with culture medium, wherein said scaffolding allows for hemopoietic cells or accessory cells to have cell to cell contacts in three dimensions.

2. The method according to claim 1, wherein the hemopoietic cells or accessory cells are mammalian hemopoietic cells or accessory cells.

3. The method according to claim 1, wherein the mammalian hemopoietic cells or accessory cells are human hemopoietic cells or accessory cells.

4. The method according to claim 1, wherein the hemopoietic cells or accessory cells are mononuclear cells.

5. The method according to claim 1, wherein the hemopoietic cells or accessory cells are selected from a the group consisting of bone marrow cells, stroma cells, peripheral blood cells, stem cells, umbilical cord cells, embryonic stem cells, peripheral stem cells, and any combination of those cells.

6. The method according to claim 1, wherein the scaffolding is selected from the group consisting of tangled fibers, porous particles, sponge, or sponge-like material.

7. The method according to claim 1, wherein the scaffolding is formed from a material selected from the group consisting of metal, glass, ceramic, plastic, hydroxyapatite, treated or untreated bone, a synthetic polymer, a natural substance, and a semisynthetic material.

8. The method according to claim 7, wherein the material is degradable.

9. The method according to claim 7, wherein the material is non-degradable.

10. The method according to claim 1, wherein the culture medium includes 1,25 dihydroxyvitamin D3, biologically active derivatives of 1, 25 dihydroxyvitamin D3, osteoclast differentiation factor, parathyroid hormone,

retinoids, thyroid hormones, leukotrienes, interleukin-1, interleukin-6, lymphotoxin, tumor necrosis factor, and colony stimulating factor-1..

11. The method according to claim 1, further comprising: identifying cells which resorb bone.

12. The method according to claim 1, further comprising: reseeding the chamber with hemopoietic cells or accessory cells.

13. Osteoclasts cultured from bone marrow cells according to claim 1.

14. Cells cultured according to the method of claim 1.

15. A method of bone synthesis or bone modification comprising growing cells cultured according to claim 1, and transplanting said cells.

16. The method according to claim 15 wherein osteoclasts and osteoblasts are transplanted together.

17. The method according to claim 15 wherein the osteoclasts and osteoblasts are cultured in series.

18. The method according to claim 15 wherein the cells are grown on a support.

19. The method according to claim 15 wherein the cells are grown without a support.

20. A method of screening for drugs which affect the ability of osteoclasts to resorb bone, comprising:

isolating hemopoietic cells or accessory cells;

culturing the hemopoietic cells or accessory cells in a container having a scaffolding covered or surrounded with culture medium, wherein said scaffolding allows for hemopoietic cells or accessory cells to have cell to cell contacts in three dimensions;

removing cultured cells; and

determining the ability of the osteoclasts to resorb bone in the presence of a test compound.

21. The method according to claim 20, wherein cultured cells are isolated osteoclasts.

22. The method according to claim 20, wherein the cultured cells are a mixture of cells from the culture.

23. The method according to claim 20, wherein the cultured cells are fractionated cells from the culture.

24. The method according to claim 20, wherein the ability of human osteoclasts to resorb bone is increased.

25. The method according to claim 20, wherein the ability of human osteoclasts to resorb bone is decreased.

26. The method according to claim 20, wherein the hemopoietic cells or accessory cells are mammalian hemopoietic cells or accessory cells

27. The method according to claim 26, wherein the mammalian hemopoietic cells or accessory cells are human hemopoietic cells or accessory cells

28. The method according to claim 20, wherein the hemopoietic cells or accessory cells are mononuclear cells

29. The method according to claim 20, wherein the hemopoietic cells or accessory cells are selected from a the group consisting of bone marrow cells, stroma cells, peripheral blood cells, and any combination of those cells.

30. The method according to claim 20, wherein the scaffolding is selected from the group consisting of tangled fibers, porous particles, sponge, or sponge-like material.

31. The method according to claim 20, wherein the scaffolding is formed from a metrial selected from the group consisting of a synthetic polymer, a natural substance, and a semisynthetic material.

32. The method according to claim 31, wherein the material is degradable.

33. The method according to claim 31, wherein the material is non-degradable.

34. The method according to claim 20, wherein the culture medium includes 1,25 dihydroxyvitamin D3 or its biologically active derivatives.

35. The method according to claim 20, further comprising: identifying osteoclasts by identifying cells which resorb bone.

36. The method according to claim 20, further comprising: reseeding the chamber with hemopoietic cells or accessory cells.

37. Isolated osteoclasts which resorb bone.

38. The osteoclasts according to claim 37, wherein the osteoclasts have calcitonin receptors.

39. The osteoclasts according to claim 37, wherein the osteoclasts express tartrate resistant acid phosphatase.

40. The isolated osteoclasts of claim 37 wherein the osteoclasts are transfected with a nucleotide sequence of a gene.

41. The isolated osteoclasts of claim 40 wherein the nucleotide sequence of a gene is heterologous to the species from which the osteoclasts are derived.

42. The isolated osteoclasts of claim 37 wherein the nucleotide sequence of a gene is native to the species from which the osteoclasts are derived.

43. The isolated osteoclasts of claim 41 or 42 wherein the nucleotide sequence codes for osteoclast differentiation factor.

44. A method of identifying genes which are related to osteoclast formation or function which comprises:

(a) isolating hemopoietic cells or accessory cells;

(b) culturing the hemopoietic cells or accessory cells in a chamber having a scaffolding covered or surrounded with culture medium, wherein said scaffolding allows for hemopoietic cells or accessory cells to have cell to cell contacts in three dimensions;

(c) altering one or more culture conditions in a test culture;

(c) determining osteoclast number and function in the test sample; and

(d) screening for the gene or genes associated with the change in osteoclast number or function in the test sample.

45. The method of claim 44 wherein osteoclast number is determined by counting TRAP positive cells.

46. The method of claim 44 wherein osteoclast function is determined by bone resorption assay.

47. The method of claim 44 wherein the screening for the gene or genes associated with change in osteoclast number or function is at least one of differential gene display, RNA arbitrarily primed (RAP)-PCR, or gene microarray analysis.

48. A method for screening for drugs which affect osteoclastogenesis which comprises:

(a) isolating hemopoietic cells or accessory cells;

(b) culturing the hemopoietic cells or accessory cells in a container or chamber having a scaffolding covered or surrounded with culture medium, wherein said scaffolding allows for hemopoietic cells or accessory cells to have cell to cell contacts in three dimensions;

(c) adding a test compound to the bioreactor,

(d) removing cultured cells; and

determining the ability of a test compound of affect osteoclastogenesis.

49. The method of claim 48 wherein the ability of a test compound to affect osteoclastogenesis is determined by osteoclast cell count, TRAP staining, bone resorption assay or a combination thereof.

50. The method of claim 48 wherein the cultured cells are isolated osteoclasts.

51. The method of claim 48 wherein the cultured cells are a mixture of cells from the culture.

52. The method of claim 48 wherein the cultured cells are fractionated from the culture.

53. The method of claim 48 wherein the effect of the test compound is to stimulate osteoclastogenesis.

54. The method of claim 48 wherein the effect of the test compound is to inhibit osteoclastogenesis.

55. The method according to claim 48, wherein the hemopoietic cells or accessory cells are mammalian hemopoietic cells or accessory cells

56. The method according to claim 48, wherein the mammalian hemopoietic cells or accessory cells are human hemopoietic cells or accessory cells

57. The method according to claim 48, wherein the hemopoietic cells or accessory cells are mononuclear cells

58. The method according to claim 48, wherein the hemopoietic cells or accessory cells are selected from a the group consisting of bone marrow cells, stroma cells, peripheral blood cells, and any combination of those cells.

59. The method according to claim 48, wherein the scaffolding is selected from the group consisting of tangled fibers, porous particles, sponge, or sponge-like material.

60. The method according to claim 48, wherein the scaffolding is formed from a metrial selected from the group consisting of a synthetic polymer, a natural substance, and a semisynthetic material.

61. The method according to claim 60, wherein the material is degradable.

62. The method according to claim 60, wherein the material is non-degradable.

63. The method according to claim 48, wherein the culture medium includes 1,25 dihydroxyvitamin D3 or its biologically active derivatives.

64. The method according to claim 48, further comprising: reseeding the chamber with hemopoietic cells or accessory cells.

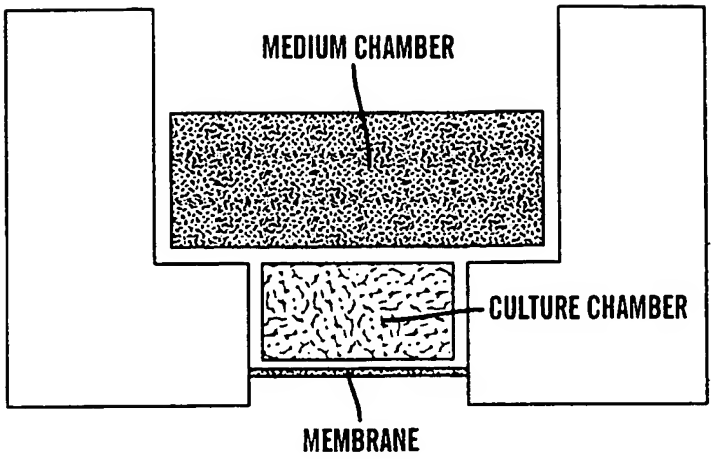
MEDIUM CHAMBER
CULTURE CHAMBER
MEMBRANE

FIG. 1a

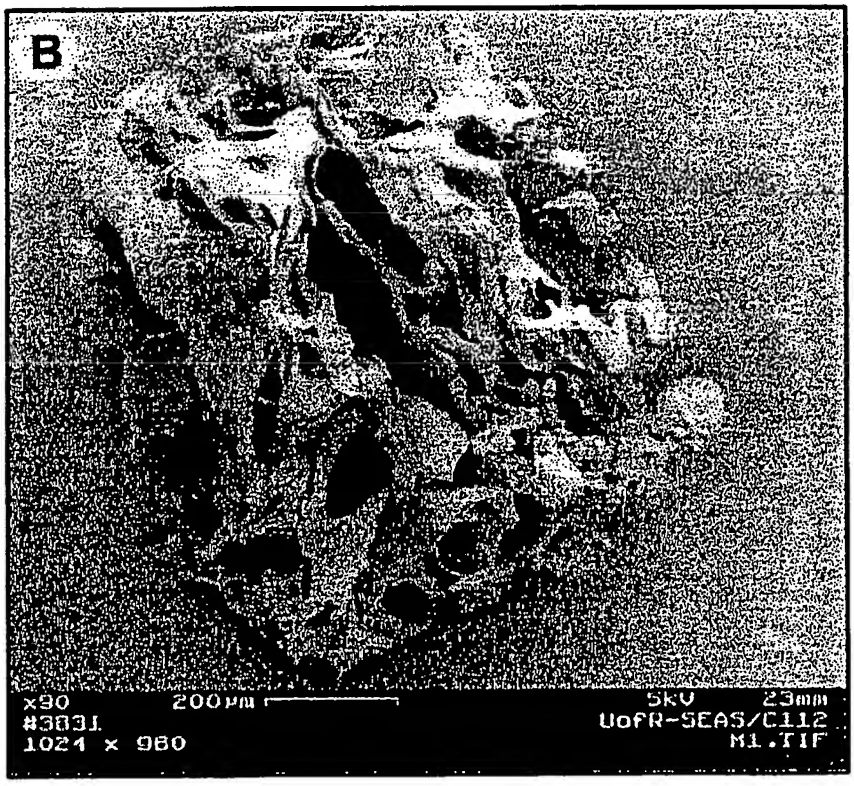
B
x90 200µm 5kV 23mm
#3831 UofR-SEAS/C112
1021 x 980 M1.TIF

FIG. 1b

FIG. 2

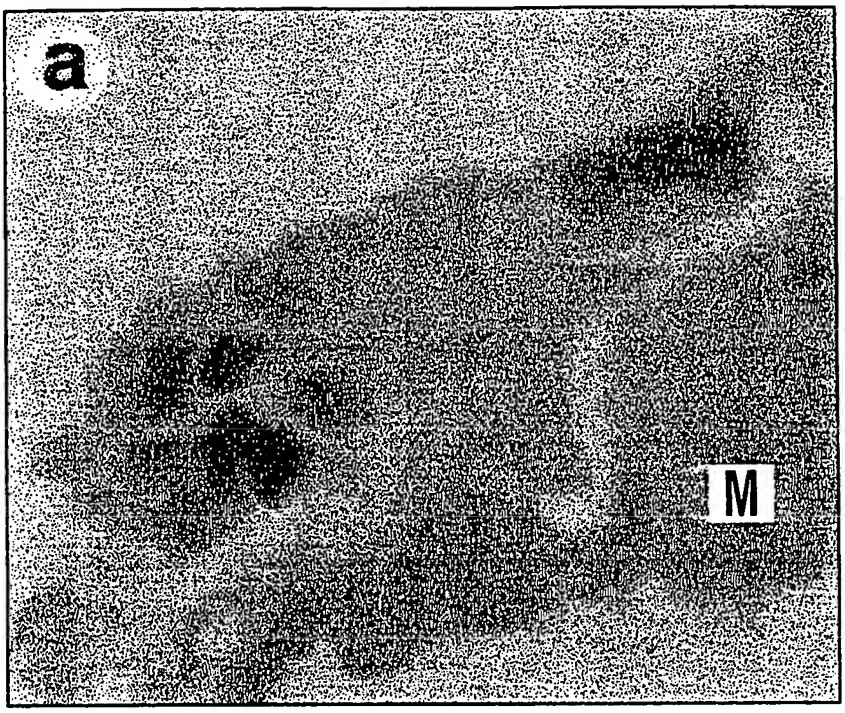

FIG. 3a

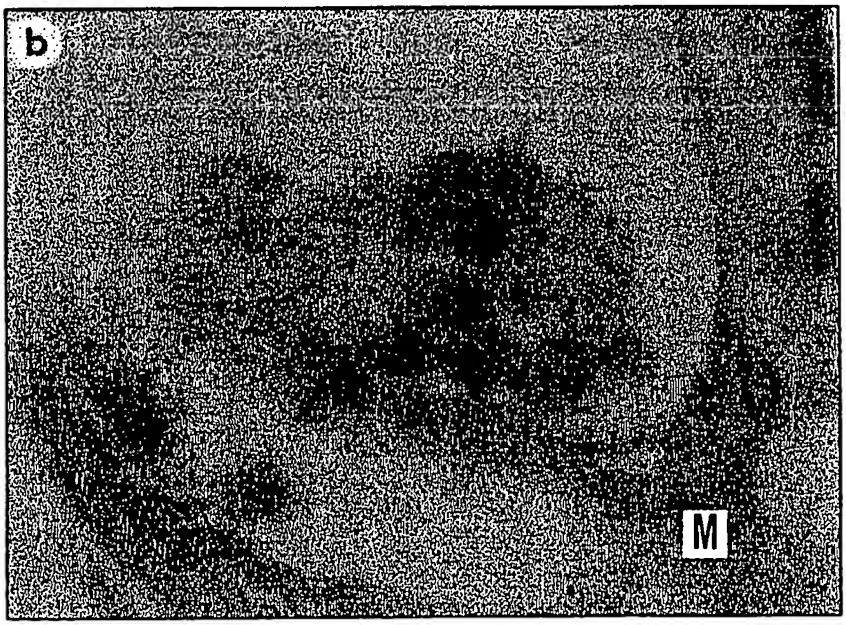

FIG. 3b

FIG. 4

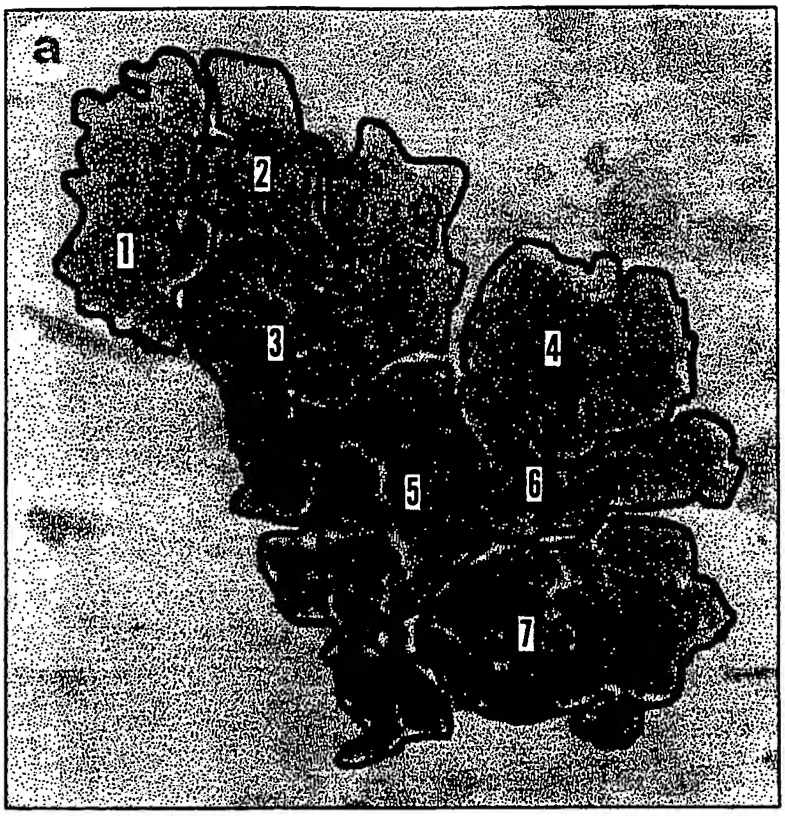

FIG. 5a

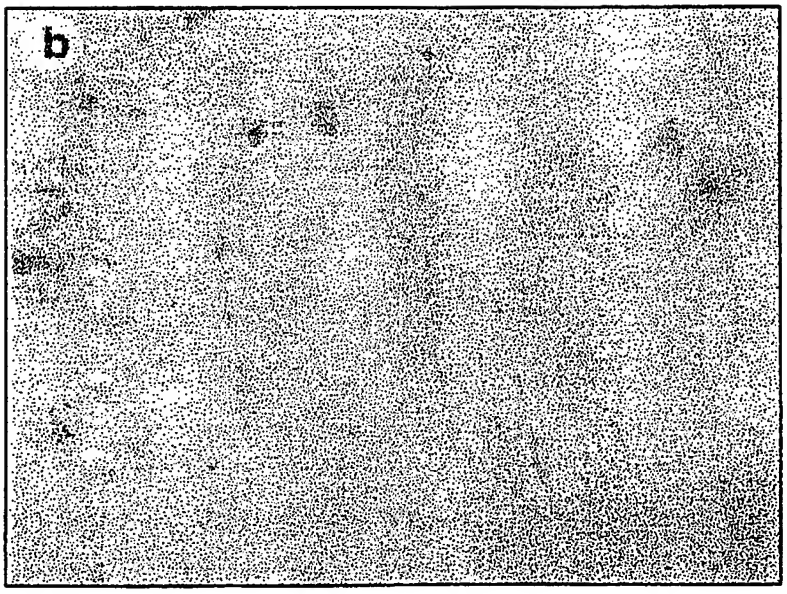

FIG. 5b

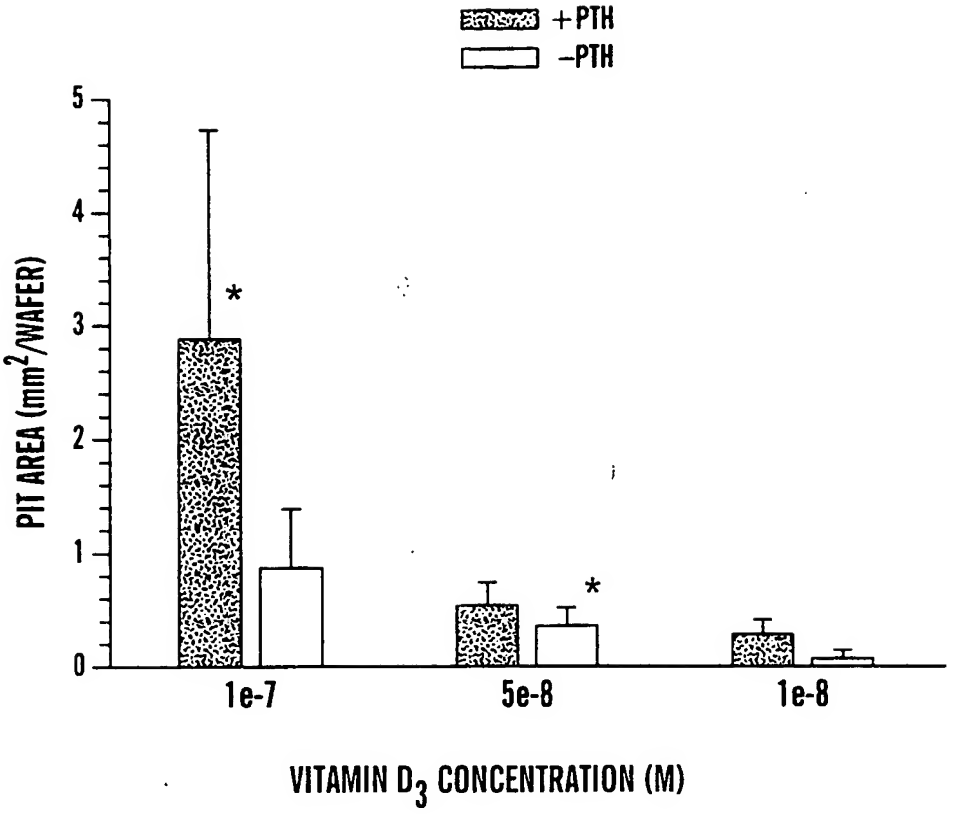
+PTH
−PTH
PIT AREA (mm²/WAFER)
0
1
2
3
4
5
*
*
1e-7
5e-8
1e-8
VITAMIN D3 CONCENTRATION (M)

FIG. 6

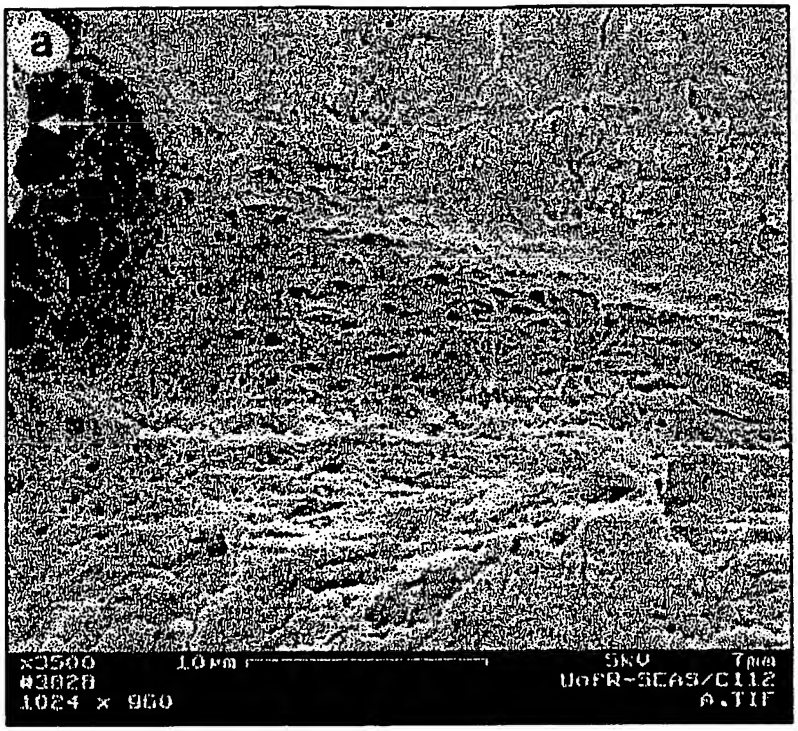
a
x3500 10μm 5kV 7mm
#3028
1024 x 960
UofPR-SEAS/C112
A.TIF

FIG. 7a

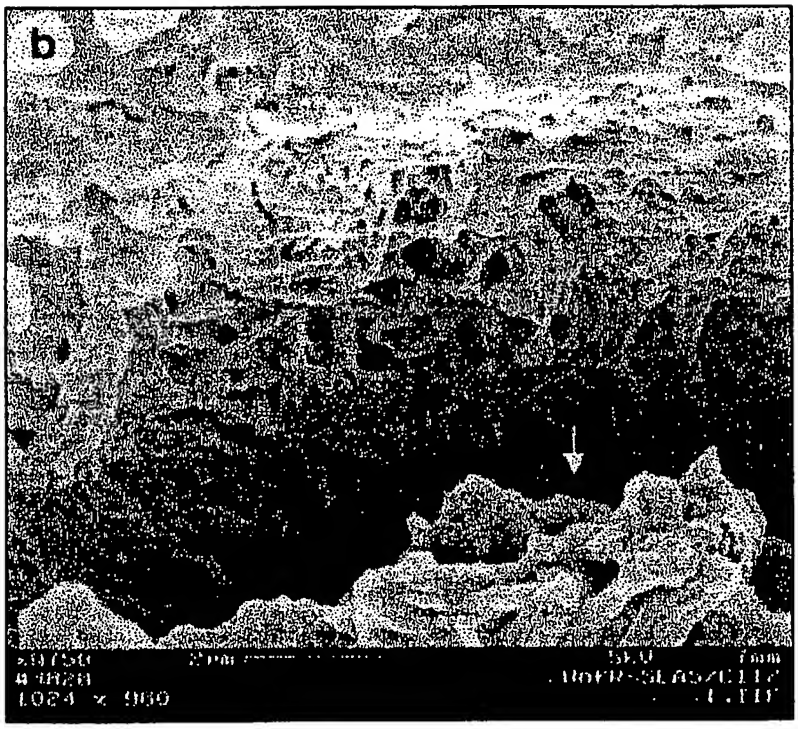
b
1024 x 960

FIG. 7b

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/22040

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 5/08; C12Q 1/00
US CL : 435/372, 4

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/372, 4, 40.5, 40.52, 325, 347, 366, 373, 375, 378, 383, 384

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,719,058 A (RODAN et al) 17 February 1998 (17.02.1998), entire document.	1-64
A	US 5,856,186 A (RODAN et al) 05 January 1999 (05.01.1999), entire document.	1-64
A, E	US 6,093,533 A (RODAN et al) 25 July 2000 (25.07.2000), entire document.	20-43, 48-64
A	ISHAUG et al. Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds. Journal of Biomed. Mat. Research. 1997, Vol. 36, pages 17-28.	1-19, 20, 26-34, 44, 48, 59-64
A	HAKEDA H.et al.The Growth and Culture of bone Cells: Osteoclastic. Principles of Bone Biology. 1996, pages 1217-1228.	1-64

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 October 2000 (20.10.2000)	14 NOV 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer George Elliott Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/22040

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.
☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/22040

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

Group I, claims 1-14, drawn to a method of culturing osteoclasts from hemopoietic cells.

Group II, claims 15-19, drawn to a method of bone synthesis or modification.

Group III, claims 20-43, drawn to a method of screening for drugs which affect bone resorbtion.

Group IV, claims 44-47, drawn to a method of identifying genes related to osteoclasts.

Group V, claims 48-64, drawn to a method for screening for drugs which affect osteoclastogenesis.

1. This International Searching Authority considers that the international application does not comply with the requirements of unity of invention (Rules 13.1, 13.2 and 13.3) for the reasons indicated below:

The inventions listed as Groups I, II, III, IV and V do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features. The unifying concept among the five groups is isolated and cultured osteoclasts. In themselves, cultured osteoclasts are not an inventive concept. They are well known and documented in the art which is evident in Hakeda et al (Principles of Bone Biology, 1996, pages 1217-1228). Hakeda teaches various ways to isolate and culture osteoclasts. In addition to the concept being well known in the art, examples applying these concepts are numerous. For example, U.S. Patent 5,766,669 amongst others applies these concepts to the practice of their inventions. Thus, Groups I-V lack a single unifying inventive concept that justifies keeping them together.

Furthermore, pursuant to 37 C.F.R. §1.475(d), the ISA/US considers that where multiple products and processes are claimed, the main invention shall consist of the first invention of the category first mentioned in the claims and the first recited invention of the other categories related thereto. Accordingly, the main invention, Group I, comprises the product, cultured osteoclast cells which are cultured according to the method set forth in claims 1-14. Thus, pursuant to 37 C.F.R. §1.475(d), the ISA/US does not consider any feature which the subsequently recited products and methods share with the main invention as a special technical feature within the meaning of PCT Rule 13.2 and that each of such products and methods accordingly defines a separate invention.

Continuation of B. FIELDS SEARCHED Item 3: EAST, BIOSIS, MEDLINE, SCISEARCH, EMBASE, DIALOG
search terms: osteoclast, osteoblast, culture, scaffold, growth, proliferation, hematopoietic, bone sythesis, resorbtion